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Published in:
Journal of Veterinary Internal Medicine

DOI:
[10.1111/jvim.12016](https://doi.org/10.1111/jvim.12016)

Publication date:
2013

Document version
Early version, also known as pre-print

Citation for published version (APA):
Liebenberg, C., Goddard, A., Wiinberg, B., Kjelgaard-Hansen, M., van der Merwe, L. L., Thompson, P. N., Matjila, P. T., & Schoeman, J. P. (2013). Hemostatic abnormalities in uncomplicated babesiosis (*Babesia rossi*) in dogs. *Journal of Veterinary Internal Medicine*, 27(1), 150-156. <https://doi.org/10.1111/jvim.12016>

Hemostatic Abnormalities in Uncomplicated Babesiosis (*Babesia rossi*) in Dogs

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Background: Babesiosis in dogs is associated with severe thrombocytopenia; yet infected dogs rarely show clinical signs of hemorrhage.

Hypothesis: Dogs with uncomplicated babesiosis have normal hemostatic capacity despite severe thrombocytopenia.

Animals: Nineteen client-owned dogs with uncomplicated babesiosis; 10 healthy controls.

Methods: A prospective, cross-sectional, observational study. Thromboelastography (TEG), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, D-dimers, and antithrombin (AT) measured in both groups. Statistical significance set at $P < .01$.

Results: Babesiosis group hematocrit and platelet count significantly lower than controls (0.29 versus 0.50 L/L; $P < .001$ and 20.0 versus 374.5×10^9 /L; $P < .001$, respectively). Except for K , no significant difference in TEG variables between groups. Hemostatic variables for babesiosis group versus controls (mean \pm SD); R : 5.9 ± 1.8 versus 4.6 ± 0.9 min ($P = .048$); K : 2.8 ± 1.1 versus 1.9 ± 0.6 min ($P = .003$); angle: 55.5 ± 11.7 versus 62.2 ± 4.1 degrees ($P = .036$); MA : 48.4 ± 9.7 versus 57.2 ± 5.2 mm ($P = 0.013$); G : 5.1 ± 1.9 versus 6.9 ± 1.5 dyn/cm² ($P = .019$); LY30 (median, range): 0 (0 – 5.7) versus 0.6% (0 – 6.1) ($P = .152$); and LY60: 0 (0 – 8.8) versus 3.1% (0 – 13.1) ($P = .012$). AT activity significantly lower (105.2 ± 16.5 versus $127.8 \pm 15.4\%$; $P = .001$). Fibrinogen concentration significantly higher in babesiosis group (5.7 ± 1.3 versus 3.0 ± 0.7 g/L; $P < .001$).

Conclusion and Clinical Importance: Despite severe thrombocytopenia, dogs with uncomplicated babesiosis did not have clinical signs of hemorrhage and TEG variables were normal, which could indicate a normocoagulable state.

Key words: Coagulation; Platelets; Thromboelastography.

Babesiosis, caused by *Babesia rossi*, is a common cause of morbidity and death of dogs in South Africa. Babesiosis in dogs can be classified as uncomplicated or complicated based on the degree of anemia and the severity of the presenting clinical signs.^{1,2} In uncomplicated babesiosis the clinical signs are mostly attributable to the degree of the anemia, whereas in complicated babesiosis the disease process is characterized by additional organ involvement.^{3,4} One of the most common hematological hallmarks of babesiosis in dogs is thrombocytopenia, which is not associated with clinical hemorrhage despite low platelet counts that would normally cause inability to maintain normal primary hemostatic function.^{5,6}

Several routine laboratory assays are traditionally utilized to diagnose and monitor abnormalities associated with hemostasis. These assays include platelet count and platelet function assays to evaluate primary

Abbreviations:

aPTT	activated partial thromboplastin time
AT	antithrombin
CBC	complete blood count
FDP	fibrin degradation products
G	global clot strength
Ht	hematocrit
K	clotting time
LY30	percent lysis at 30 minutes
LY60	percent lysis at 60 minutes
MA	maximal amplitude
PCR	polymerase chain reaction
PT	prothrombin time
RLB	reverse line blot
R	reaction time
SIRS	systemic inflammatory response syndrome
TEG	thromboelastography
α	angle

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Submitted September 29, 2011; Revised August 14, 2012; Accepted October 10, 2012.

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10.1111/jvim.12016

hemostasis and prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrin(ogen) degradation products (FDP), D-dimer concentration, antithrombin (AT) activity, and fibrinogen concentration to evaluate secondary hemostasis and fibrinolysis.⁷ Limitations associated with the majority of these assays are their insensitivity to identify the early onset of hemostatic abnormalities, as well as the fact that each assay only evaluates a specific part of the hemostatic pathway.

Systemic inflammatory response syndrome (SIRS) is a well-described consequence of babesiosis⁸ and one of the key components of SIRS is the activation of an acute phase response with markedly increased plasma

concentrations of fibrinogen.^{9,10} Several studies have reported that inflammation can activate hemostasis.^{11,12} The etiology has been extensively studied and the interactions between these 2 systems are best illustrated with the cell-based model of hemostasis.¹³ Few assays are available that accurately reflect the interactions between inflammation and hemostasis, but thromboelastography (TEG) incorporates the interactions of all the important intravascular components of the hemostatic system, except the endothelium, and is believed to be a closer approximation of hemostasis as it occurs in vivo.¹⁴ TEG is influenced by hematocrit (Ht), platelet count, platelet function, fibrinogen concentration, and AT activity.^{14–16} The platelet function is measured as the overall clot strength (*MA*), which is primarily dependent on the cross binding of platelets and fibrinogen. Importantly, with respect to the low platelet count seen in babesiosis, platelet concentration affects the *MA* and several studies have reported that a decreased platelet count leads to hypocoagulable thromboelastograms.^{17–19} Equally important is the data from in vitro studies in people which have shown that *MA* can be normalized in severe thrombocytopenia, with platelet counts as low as $10 \times 10^9/L$, by addition of fibrinogen.²⁰ To the authors' knowledge, TEG is one of the only methods available to measure the interaction between platelets and fibrinogen in whole blood—an interaction which might well be crucial for the absence of clinical hemorrhage in the face of severe thrombocytopenia, seen in babesiosis.

The aim of this study was to describe the thromboelastograms in uncomplicated babesiosis in dogs and compare them with those of normal, healthy control dogs. We hypothesized that these dogs would have a normal hemostatic capacity, despite the severe thrombocytopenia, and that this could be measured with TEG.

Materials and Methods

Study Design

This study was approved by the Animal Use and Care Committee of the University of Pretoria. It was performed as a prospective, cross-sectional, observational study. A total of 29 dogs were enrolled in this study: 19 client-owned dogs diagnosed with uncomplicated babesiosis and 10 healthy client-owned dogs. Only uncomplicated cases were included in an attempt to standardize the study and limit variability. The control dogs were selected to match (age and sex) that of the dogs with babesiosis. Owner consent was obtained for all the cases for enrollment in this study.

Inclusion and Exclusion Criteria

Eligible dogs were of any breed and either sex, provided they were ≥ 6 months of age, weighed more than 6 kg, and were naturally infected by *B. rossi* parasites. *Babesia rossi* infection was confirmed and coinfection with *Ehrlichia canis* was ruled out using a Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB). None of the dogs that were included showed any clinical evidence of a hemorrhagic tendency. Dogs were excluded if they had any clinical or laboratory evidence of complications associated with babesiosis, which included acute kidney failure,

neurological signs, acute respiratory distress syndrome, hemoconcentration, secondary immune mediated hemolytic anemia, and icterus. Dogs were also excluded if any concurrent inflammatory disease conditions, any known cardiac disease, any known neoplastic disease, any obvious infections or wounds, or any signs of trauma (such as fractures, contusions, and wounds indicating a motor vehicle accident or fighting) were present. Treatment with any medication known to interfere with normal hemostasis including prednisolone, aspirin, nonsteroidal anti-inflammatory drugs, or heparin products either at presentation or 4 weeks prior to presentation were also reason for exclusion. The controls included 10 healthy, client-owned dogs, admitted for routine ovariohysterectomy, castration, or blood donation. The controls were deemed healthy based on history, a full clinical examination which included a peripheral blood smear, as well as PCR and RLB to rule out blood-borne parasites.

Sampling

Prior to any treatment, a serum sample (3 mL vacutainer tube^a), sodium citrate sample (3 mL vacutainer tube), and EDTA sample (3 mL vacutainer tube) were collected from the jugular vein of each dog, with a 21-gauge venoject needle by careful venipuncture with minimum stasis. Sufficient blood was collected in the citrate tube to ensure a 1 : 9 ratio of 3.2% trisodium citrate and blood. The blood samples were collected in the order described above. The EDTA sample was used to determine the platelet count and Ht. The TEG analysis was performed on the sodium citrate sample 30 minutes after collection. The remaining citrated sample was then centrifuged at $2100 \times g$ for 8 minutes, after which the plasma was harvested and stored at -80°C . The coagulation profile analyses were performed as a batch within 4 months of collection and included PT, aPTT, D-dimer, AT and fibrinogen. Studies in people and dogs have reported that coagulation proteins in frozen plasma remain stable between 6 and 24 months at -70°C .^{21,22}

Thromboelastography Analysis

The TEG analyses were performed 30 minutes after blood sampling using a thromboelastograph^b according to a previously published method.¹⁶ Several values are derived from the thromboelastogram. The reaction time (*R*) or precoagulation time is related to the plasma clotting factors and inhibitor activity. The clotting time (*K*) represents the rate of thrombin generation and is affected by clotting factors, fibrinogen, and platelets. Angle (α) represents the rate of fibrin build-up and cross linking similar to *K* and is affected by the same factors. The maximal amplitude (*MA*) represents the strength of the fibrin clot and is affected by fibrin and fibrinogen concentration, platelet count and function, thrombin concentration, factor XIII and Ht. The *MA* is also a measure of clot stiffness and may be used to derive the global clot strength (*G*), a measure of the overall coagulant state. The *G* value is derived by the calculation: $G = 5000 \times MA / (100 - MA) \text{ dyn/cm}^2$. Using the *G* value one can characterize thromboelastograms as hyper-, normo-, or hypocoagulable.¹⁴ Previous studies have reported $G < 3.2 \text{ dyn/cm}^2$ as hypocoagulable and $> 7.2 \text{ dyn/cm}^2$ as hypercoagulable.²³ The LY30 and LY60 values indicate the degree of fibrinolysis at 30 and 60 minutes after *MA* is reached.²⁴

Coagulation Assays

A complete blood count (CBC) was performed to determine the Ht and platelet count of each dog.^c The PT and aPTT assays as well as the fibrinogen were performed on an automated hemostasis

analyzer^d with specific reagent kits for each analysis.^{c,f,g} The D-dimer concentration was determined using an immunometric flow-through principle.^h AT activity in the plasma was measured utilizing a thrombin dependent chromogenic substrate assayⁱ on an automated analyzer. All assays were calibrated according to the manufacturers' recommendations for human purposes. Commercially available human control reagents for the respective assays were analyzed as internal controls together with pooled plasma from 5 healthy dogs.

DNA Extraction and PCR

DNA was extracted from 200 μ L of each whole blood sample using the QIAmp[®] blood and tissue extraction kit^j according to the manufacturer's instructions. Molecular diagnosis of *B. rossi* and exclusion of other *Babesia* species, *Ehrlichia*, and *Anaplasma* species was performed using PCR and RLB assay as previously described.^{25,26}

Statistical Analysis

Data were analyzed using Stata 12.1 statistical software^k The normality assumption was evaluated using the Shapiro-Wilk test and equality of variances was evaluated using Levene's test. Differences between the babesiosis group and the control group were tested using the Students *t*-test for normally distributed variables or the Mann-Whitney-*U*-test for non-normally distributed variables. Multiple linear regression analyses were performed within the babesiosis group to estimate the association of platelet count, Ht and fibrinogen with the various TEG variables. For this purpose, platelet count and Ht were log-transformed to achieve normality. The regression models were evaluated using residual versus fitted plots and normal probability plots of residuals. Significance was set at $P < .01$ to reduce the risk of a family wise Type I error rate in this multiple comparison setting.

Results

The age for the babesiosis group was 6 years (7 months-11 years; median, range) and the group consisted of 11 females and 8 males. Breeds included

four rottweilers, three Boerboels, two cross breed dogs, two Jack Russell terriers, two dachshunds and one of each of the following, fox terrier, toy pomeranian, Labrador retriever, Staffordshire terrier, Chow-chow and maltese. The age for the control group was 3.25 years (6 months-6 years) and it consisted of 6 females and 4 males. Breeds included three beagles, two Boerboels, two German shepherd dogs and one of each of the following, Rhodesian ridgeback, Bouvier des Flanders and a cross breed.

Except for the mean *K*, which was longer in the babesiosis than in the control group, there were no significant differences in the TEG variables (mean *R*, angle, *MA*, *G*, and median LY30 and LY60) between the groups. Three dogs with babesiosis had *G* values in the hypercoagulable range (>7.2 dyn/cm²) according to previous studies²³ i.e., 9.25, 8.50, and 7.65 dyn/cm², respectively. The hematocrit and platelet count, and AT activity were significantly lower in the babesiosis group compared to the control group. No significant platelet aggregation, which could have contributed to the low platelet count, was reported in the samples. The fibrinogen concentration was significantly higher in dogs with babesiosis compared to the control group, but the median D-dimer concentration was not significantly different. PT was not significantly different between the two groups ($P = .076$), but the aPTT was significantly prolonged in the babesiosis group ($P = .001$). (Table 1).

Within the dogs with babesiosis, multiple regression analysis revealed a significant positive log-linear association between platelet count and *MA* ($r^2 = 25.19$; $P < .001$) and platelet count and *G* ($r^2 = 5.30$; $P < .001$); and a significant negative log-linear association between platelet count and *K* ($r^2 = -2.10$; $P = .002$). (Table 2) These are shown at the univariable level in the scatter plots of platelet count, Ht and fibrinogen versus *G*. (Fig. 1A-C) There were no significant associations

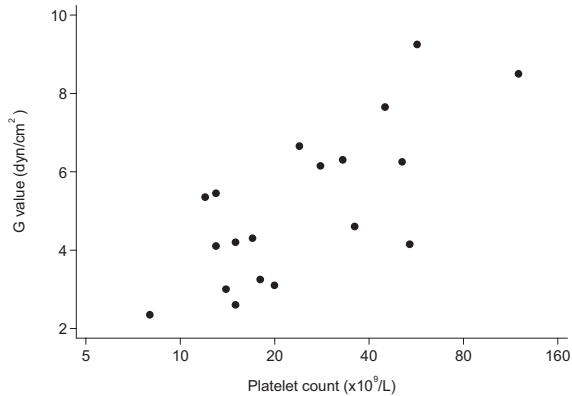
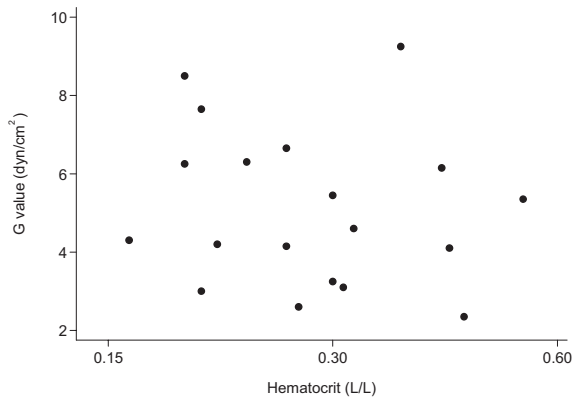
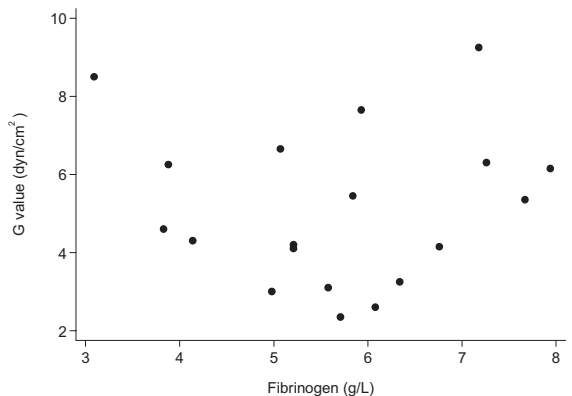
Table 1. Mean (range) of the coagulation measures for the dogs with uncomplicated babesiosis and the healthy control groups with respective *P*-values. Median (range) shown for hematocrit, platelet count, LY30, LY60, PT and D-dimer concentration.

Parameter (unit)(Reference range)	Babesiosis GroupMean/Median (Range)	Control GroupMean/Median (Range)	<i>P</i> -Value
Plt (10^9 /L) (200–500)	20.0 (8.0–120.0)	374.5 (224.0–678.0)	<.001
Ht (L/L) (0.37–0.55)	0.29 (0.16–0.54)	0.50 (0.38–0.59)	<.001
<i>R</i> – time (minutes) (3–9)	5.9 (2.0–8.5)	4.6 (3.7–6.0)	.048
<i>K</i> – time (minutes) (2–8)	2.8 (1.2–4.4)	1.9 (1.5–2.3)	.003
Angle (degrees) (27–59)	55.5 (33.6–71.3)	62.2 (56.6–70.2)	.036
<i>MA</i> (mm) (39–59)	48.4 (31.6–64.9)	57.2 (48.7–65.9)	.013
<i>G</i> (dyn/cm ²) (3.2–7.2)	5.1 (2.4–9.3)	6.9 (4.9–10.0)	.019
LY30 (%) (0–2)	0.0 (0.0–5.7)	0.6 (0.0–6.1)	.152
LY60 (%) (0–8)	0.0 (0.0–8.8)	3.1 (0.0–13.1)	.012
PT (seconds) (<6.9)	6.5 (5.5–8.1)	6.9 (6.3–11.7)	.076
aPTT (seconds) (<12.9)	13.3 (10.4–15.5)	11.3 (9.2–12.5)	.001
AT (%) (80–135)	105.2 (70.4–143.5)	127.8 (109.4–159.4)	.001
Fibrinogen (g/L) (2–4)	5.7 (3.1–7.9)	3.0 (1.9–4.4)	<.001
D-dimers (mg/L) (<0.5)	0.3 (0.1–1.0)	0.1 (0.1–0.3)	.015

Platelet count (Plt); Hematocrit (Ht); Reaction time (*R*); Clotting time (*K*); Angle (α);Maximal amplitude (*MA*); Global clot strength (*G*); Percent lysis at 30 minutes (LY30); Percent lysis at 60 minutes (LY60); activated partial thromboplastin time (aPTT); Prothrombin time (PT); Antithrombin (AT).

Table 2. Summary of results of multiple linear regression models of the association of log platelet count, log Ht, and fibrinogen with TEG variables (*R*, *K*, angle, *MA*, and *G*) in uncomplicated *Babesia*-infected dogs.

Predictors	Outcome [Regression Coefficient (<i>P</i> -value)]				
	<i>R</i>	<i>K</i>	Angle	<i>MA</i>	<i>G</i>
log ₁₀ Plt	0.07 (0.965)	−2.10 (0.002)	14.30 (0.113)	25.19 (<0.001)	5.30 (<0.001)
log ₁₀ Ht	2.16 (0.577)	3.28 (0.025)	−25.90 (0.227)	−0.93 (0.948)	1.23 (0.677)
Fibrinogen	0.38 (0.349)	−0.06 (0.674)	−0.87 (0.689)	0.95 (0.527)	0.21 (0.496)

A**B****C****Fig 1.** Scatter plots illustrating the associations of (A) platelet count, (B) Ht, and (C) fibrinogen with *G*.

between Ht or fibrinogen and any of the TEG variables. Residual plots showed no obvious deviations from the assumptions of normality, linearity or homoscedasticity for any of the models. AT was not included in these models since the mean AT was still well above reference range despite being significantly lower in the babesiosis group ($P = .001$).

Discussion

This study demonstrated that, except for *K*, there was no significant difference in any of the TEG variables between the babesiosis group with a median platelet count of $20 \times 10^9/L$ and a control group consisting of healthy dogs with normal platelet counts. One of the hematological hallmarks of babesiosis in dogs, caused by *B. rossi*, is thrombocytopenia, which is not associated with clinical hemorrhage despite platelet counts that would normally cause an inability to maintain normal primary hemostatic function.^{5,6} TEG thus demonstrated that dogs with severe thrombocytopenia secondary to uncomplicated *B. rossi* infection may have normal overall hemostatic function, which correlates with the clinical presentation.

An adequate number of normally functioning platelets are vital for primary hemostasis and platelet counts below $66 \times 10^9/L$ have been shown to affect the TEG variables *K*, *MA*, and *G* in studies conducted in both people and dogs.^{17,19,23,27} However, despite a median platelet count of $20 \times 10^9/L$, the thromboelastograms of the babesiosis group in this study did not have a mean *K*, *MA*, or *G* below the reference interval. In fact, 3 *Babesia*-infected dogs with severe thrombocytopenia had an increased *G* ($G > 7.2 \text{ dyn/cm}^2$), suggesting hypercoagulability.²³ This study showed that there was a significant positive association between platelet count and *MA* and platelet count and *G*, and a significant negative association between platelet count and *K*, indicating that the lower the platelet count, the relatively more hypocoagulable the thromboelastogram. However, these findings were of no clinical significance since the mean values for the TEG variables in the babesiosis group remained within the normal reference ranges. The authors believe that the most likely explanation for this finding is due to the effect of marked platelet activation, subsequent to the systemic inflammation, providing a procoagulant membrane surface upon which coagulation factors assemble. Thus, the babesiosis group exhibited a relative hypercoagulable state, compared to what is normally seen in dogs with such

extremely low platelet counts, which explains the normal TEG variables. Both *B. canis* and *B. rossi* cause a systemic inflammatory response with increased C-reactive protein, hyperfibrinogenemia, thrombocytopenia, and leukopenia.^{8,9,28} Inflammation promotes coagulation and thrombin can in turn cause inflammation.²⁹ Although platelets are activated by many stimuli, thrombin is the most potent platelet activator.³⁰ Platelet activation and the coagulation cascade collectively determine the hemostatic activity of plasma—both are intertwined and thrombin is instrumental in each process.^{13,31}

This study showed that there were no significant associations between Ht and any of the TEG variables. In uncomplicated babesiosis the clinical signs are mostly attributable to the degree of the anemia.³ Since the thromboelastogram is influenced by all components of coagulation, the anemia in dogs suffering from babesiosis has to be considered when evaluating the results. Studies have reported that a decreased red cell mass can lead to hypercoagulable tracings.^{32–34} However, it is still unclear whether this hypercoagulability due to decreased red cell mass is a true reflection of in vivo hypercoagulability.^{32–35} The anemia present in canine babesiosis is due to hemolysis, caused by the intra-erythrocytic parasite.³⁶ The local disruption of the red blood cells leads to the exposure of intracellular stromal material, especially phospholipids, which are expected to trigger the coagulation system.^{37,38} Yet, ironically, 2 other studies evaluating in vitro hemolysis (either mechanically induced or by freeze-thawing) revealed decreased *MA* and *G* values consistent with hypocoagulability.^{39,40}

The significantly higher mean fibrinogen concentration in the babesiosis group as compared to the controls, is consistent with an acute phase response.²⁸ Platelet surfaces are densely populated with receptors of which the glycoprotein IIb/IIIa complex (GPIIb/IIIa) is the most prominent.⁴¹ In people, in vitro TEG studies have reported that even with severe thrombocytopenia, thrombin-activated platelets bind to large amounts of fibrinogen via GPIIb/IIIa receptors, resulting in increased clot strength.^{20,42} Further evidence for the concomitant activation of coagulation was supported by the mean AT activity, which was significantly lower in the babesiosis group. During severe inflammation AT activity is decreased due to impaired synthesis as result of a negative acute phase response, degradation by granulocytic elastase, consumption as a consequence of ongoing thrombin formation and impairment due to the reduced availability of glycosaminoglycans.^{11,43} The D-dimer value was not significantly different in the babesiosis group compared to the controls and the D-dimer values of the babesiosis group mostly remained within the normal range. The TEG variables for fibrinolysis (LY30 and LY60) were also not significantly different and the babesiosis group fell within the normal ranges, indicating that there was no measurable activation of the fibrinolytic system.

The PT was not significantly different between the babesiosis and control group; however, the mean

aPTT was significantly prolonged in the babesiosis group compared to the control group. This study did not further investigate the finding of a prolonged aPTT in the presence of normal TEG variables. The clinical relevance of this finding is questionable as the difference was small with overlap between the groups. This assay is also subject to large biological variation and laboratory variance. A prolonged aPTT has been reported in dogs with IMHA, in conjunction with hypercoagulable TEG values.⁴⁴ Prolonged aPTT has also been reported in Bernese Mountain Dogs with normal thromboelastograms and it was speculated that this could be due to antiphospholipid antibodies.⁴⁵ The influence of antiphospholipid antibodies on the aPTT values in our study is unknown; however, it is likely that they play a role, since the anemia seen in dogs with babesiosis is due to hemolysis, and the local disruption of the red blood cells leads to the exposure of intracellular stromal material, especially phospholipids, which could trigger an immune response.^{37,38}

Limitations to this study included the small sample size and the broad Ht range of the babesiosis dogs, which makes it difficult to draw inferences on the effect of Ht on the TEG variables. Consequently, future studies should be conducted in which larger numbers of babesiosis cases are categorized into several Ht ranges.

In summary, our study has shown that dogs suffering from uncomplicated babesiosis, caused by *B. rossi*, have normal TEG variables in the face of severe thrombocytopenia, anemia, and hyperfibrinogenemia. This finding could indicate a normocoagulable state in these patients due to a net result of the opposing effects on TEG of these abnormalities. However, the normocoagulable state could also be as a result of the inflammatory process that is present in babesiosis, causing marked platelet activation which will bind to the high concentration of fibrinogen, resulting in increased clot strength. Further research is required to investigate this phenomenon in more detail.

Footnotes

- ^a BD Vacutainer tube, S.A. Scientific, Bryanston, South Africa
- ^b TEG® 5000 Thrombelastograph® Hemostasis System Haemoscope, Pro-Gen Diagnostics (Pty) Ltd, South Africa
- ^c Advia 2120 Siemens, South Africa
- ^d ST art® 4 analyzer DiagnosticaStago, Roche, South Africa
- ^e Neoplastine® CI Plus reagent kit DiagnosticaStago reagent kit
- ^f C.K. Prest® reagent kit DiagnosticaStago reagent kit
- ^g Sta-Fib 2 reagent kit, DiagnosticaStago
- ^h D-dimer single test, Nycocard Reader, ILEX South Africa (Pty) Ltd
- ⁱ Cobas Integra 400 plus, AT III cassette reagent, DiagnosticaStago
- ^j QIAmp® blood and tissue extraction kit, Qiagen, Hilden, Germany
- ^k StataCorp, College Station, TX

Acknowledgments

Pro-Gen Diagnostics (Pty) LTD South Africa for sponsoring the thromboelastography analyzers.

Research: The research project was conducted at the Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria, Onderstepoort. All coagulation assays and TEGs were performed by the Clinical Pathology Laboratory, Department of Companion Animal Clinical Studies.

Grants or presentations: The work was presented as an abstract at the 20th Annual Congress of the European College of Veterinary Internal Medicine 2010, Toulouse, France.

Conflict of Interest Declaration: Authors disclose no conflict of interest.

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